Yes-associated Protein 65 Localizes p62$^{c\text{-}Yes}$ to the Apical Compartment of Airway Epithelia by Association with EBP50

Peter J. Mohler,* Silvia M. Kreda,‡ Richard C. Boucher,‡ Marius Sudol,§ M. Jackson Stutts,‡ and Sharon L. Milgram*

*Department of Cell and Molecular Physiology, ‡The Cystic Fibrosis/Pulmonary Research and Treatment Center, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599; and §Department of Biochemistry, Mount Sinai Medical School, New York University, New York, NY 10029

Abstract. We recently showed that the COOH terminus of the cystic fibrosis transmembrane conductance regulator associates with the submembranous scaffolding protein EBP50 (ERM-binding phosphoprotein 50 kD; also called Na^+/H^+ exchanger regulatory factor). Since EBP50 associates with ezrin, this interaction links the cystic fibrosis transmembrane conductance regulator (CFTR) to the cortical actin cytoskeleton. EBP50 has two PDZ domains, and CFTR binds with high affinity to the first PDZ domain. Here, we report that Yes-associated protein 65 (YAP65) binds with high affinity to the second EBP50 PDZ domain. YAP65 is concentrated at the apical membrane in airway epithelia and interacts with EBP50 in cells. The COOH terminus of YAP65 is necessary and sufficient to mediate association with EBP50. The EBP50–YAP65 interaction is involved in the compartmentalization of YAP65 at the apical membrane since mutant YAP65 proteins lacking the EBP50 interaction motif are mislocalized when expressed in airway epithelial cells. In addition, we show that the nonreceptor tyrosine kinase c-Yes is contained within EBP50 protein complexes by association with YAP65. Subapical EBP50 protein complexes, containing the nonreceptor tyrosine kinase c-Yes, may regulate apical signal transduction pathways leading to changes in ion transport, cytoskeletal organization, or gene expression in epithelial cells.

Key words: EBP50 • NHERF • YAP65 • Src family kinase • scaffolding protein

THE vectorial transport of ions and water across an intact epithelium requires selective sorting of receptors, ion channels, and transporters to apical or basolateral cell surfaces. The coordinate regulation of ion transport is thought to depend upon the compartmentalization of regulatory proteins at the appropriate plasma membrane domain. Such compartmentalization may be accomplished by binding of receptors, effectors, and regulatory proteins to submembranous scaffolding proteins with multiple protein–protein interaction domains (Pawson and Scott, 1997).

The presence of one or more PDZ domains is emerging as a hallmark feature of submembranous scaffolding proteins. PDZ domains were first identified as conserved 90–100 amino acid sequences within the postsynaptic density protein PSD95, the *Drosophila* tumor suppressor dlg-A, and the tight junction protein ZO-1 (Woods et al., 1996; Tejedor, 1997; Fanning et al., 1998). PDZ domains mediate interaction with the COOH termini of proteins terminating in consensus PDZ binding sequences (Songyang et al., 1997; Sudol, 1998). In addition, PDZ domains may dimerize (Brenman et al., 1996; Xu et al., 1998) or participate in unconventional interactions involving internal sequences within the PDZ-binding proteins (Shieh and Zhu, 1996). Studies of PSD95 and its associated receptors and ion channels clearly establish the importance of PDZ domains in the sorting of proteins to specialized membrane domains and the coordination of signal transduction in cells (Niethammer et al., 1996; Sheng and Kim, 1996).

A number of different PDZ-containing scaffolding proteins are expressed at basolateral or apical cell surfaces in epithelia (for review see Fanning and Anderson, 1999). For example, hDlg, CASK/LIN-2, and syntrophin are restricted to lateral and/or basal surfaces (Lue et al., 1996; Cohen et al., 1998; Kachinsky et al., 1999). In contrast, ERM-binding phosphoprotein 50 (EBP50;[1] also called

Address correspondence to Sharon L. Milgram, Cell and Molecular Physiology, University of North Carolina at Chapel Hill, CB7545, Chapel Hill, NC 27599. Tel.: (919) 966-9792. Fax: (919) 966-6927. E-mail: milg@med.unc.edu

1. *Abbreviations used in this paper:* CFTR, cystic fibrosis transmembrane conductance regulator; EBP50, ERM-binding phosphoprotein 50 kD; E3KARP, NHE3 kinase A regulatory protein; ERM, ezrin-radixin-moesin; NHERF, Na^+/H^+ exchanger regulatory factor; NRTK, nonreceptor tyrosine kinase; PKA, protein kinase A.

Na^+/H^+ exchanger regulatory factor [NHERF]) is preferentially localized at the apical membrane (Reczek et al., 1997; Short et al., 1998). The rabbit orthologue of EBP50 was cloned as a cofactor necessary for protein kinase A (PKA)–mediated inhibition of Na^+/H^+ exchange (Weinman et al., 1995; Yun et al., 1997), and was therefore named NHERF. The human NHERF orthologue was purified from the placenta and named EBP50 based on its ability to bind members of the ezrin-radixin-moesin (ERM) family (Reczek et al., 1997). EBP50 is 358 amino acids in length; amino acids 11–97 and 150–237 are PDZ domains that share ~75% identity (see Fig. 1).

The first PDZ domain of EBP50 binds with high affinity to the COOH terminus of the cystic fibrosis transmembrane conductance regulator (CFTR) (Hall et al., 1998a; Short et al., 1998; Wang et al., 1998). CFTR functions as an apical membrane Cl^- channel regulated by multiple kinases and phosphatases (for reviews see Ma and Davis, 1998; Gadsby and Nairn, 1999). CFTR is also known to affect the function of several transporters and ion channels in the apical membrane (for review see Schwiebert et al., 1999). In fact, CFTR is required for cAMP-mediated inhibition of NHE3 (Clarke and Harline, 1996), a protein known to associate with EBP50 (Weinman et al., 1995; Yun et al., 1997; Lamprecht et al., 1998; Yun et al., 1998). Similarly, CFTR prevents cAMP-mediated stimulation of the amiloride-sensitive epithelial Na^+ channel in some epithelia (Stutts et al., 1995). Although the function of the CFTR–EBP50 interaction is not yet understood, EBP50 may be required for the efficient targeting or regulation of CFTR, or for CFTR-mediated regulation of other ion channels and transporters.

As an approach to understand the significance of the CFTR–EBP50 interaction, we sought to identify proteins that bind the second PDZ domain of EBP50. Screening of random peptide libraries established that peptides terminating in the amino acid sequence TRL bound with high affinity to PDZ1, whereas peptides terminating with TWL bound with high affinity to PDZ2 (Wang et al., 1998). Therefore, we screened protein databases and identified Yes-associated protein 65 (YAP65) as a possible EBP50 PDZ2-binding protein. YAP65 is a modular adaptor protein known to associate with the nonreceptor tyrosine kinase $p62^{c\text{-}Yes}$ (c-Yes) in vitro (Sudol, 1994). Therefore, we studied the interaction between EBP50, YAP65, and c-Yes in airway epithelia. Our data suggest that EBP50 recruits YAP65 and c-Yes to the apical compartment, where c-Yes is well positioned to regulate important cellular processes that occur at this cell surface.

Materials and Methods

Peptides and Antibodies

The following wild-type peptides were synthesized at the UNC Peptide Facility: WW domain of human YAP65 (VPLPAGWEMAKTSSGQRYFLNHIDQTTTWQDPRK; YAP WW), the COOH-terminal 10 amino acids of YAP65 (YAPwt), and the COOH-terminal 10 amino acids of human CFTR (CFTRwt; see Fig. 2 B). Mutant peptides (YAPmut and CFTRmut), where the final four amino acids of YAP65 or CFTR were changed to glycine, were also synthesized. All peptides contained an amino-terminal biotin and a 4–amino acid spacer (SGSG) and were purified by high pressure liquid chromatography.

Rabbit antibodies directed against amino acids 142–432 of chicken YAP65 were described previously (Sudol, 1994). Mouse anti-Src antisera was provided by Dr. Patricia Maness (University of North Carolina). Commercial antisera were obtained from the following sources: mouse anti-Yes and mouse anti-ezrin from Transduction Laboratories; rabbit anti-Yes from Upstate Biotechnology, mouse anti-Src from Santa Cruz Biotechnology, and rat anti-ZO-1 and mouse anti-GFP from Chemicon International, Inc.

EBP50 antisera were generated in rabbits using hexahistidine-tagged full-length EBP50 as an immunogen. The bacterial expression plasmid pET.EBP50 was generated by subcloning the full-length EBP50 cDNA from pGEX.EBP50 (Short et al., 1998) to pET28c (Novagen, Inc.). Hexahistidine-tagged EBP50 protein was purified on a Ni^{2+} affinity resin, and the purified protein was used to immunize two New Zealand white rabbits (Covance Laboratories). EBP50 antisera were analyzed by Western blot analysis using CalU3 cell lysates known to contain high levels of EBP50. Before immunolocalization studies, complement proteins were removed from the whole serum by incubation with DEAE-blue dextran (Pierce Chemical Co.). In addition, some experiments were performed with EBP50 antisera provided by Dr. Anthony Bretscher (Cornell University), and similar results were obtained.

Plasmids for Bacterial and Mammalian Expression

pGEX plasmids encoding GST fusions of EBP50, PDZ1, and PDZ2 were described (Short et al., 1998). For in vitro translation experiments, pCDNA3.CFTR-CT encoding the COOH-terminal 330 amino acids of human CFTR (amino acids 1,151–1,480) was generated by PCR using pBQ.CFTR as template. PCR products were digested with the appropriate enzymes and ligated into the polylinker of pET.28c digested with the same enzymes (Novagen, Inc.). For in vitro translation and mammalian expression, full-length human YAP65 and a mutant human YAP65 (YAP65/−4) were generated by PCR. The mutant YAP65/−4 construct has an engineered premature stop codon after amino acid 450, resulting in expression of a YAP65 protein lacking the final four amino acids. PCR products were digested with the appropriate enzymes and ligated into the polylinker of pCDNA3.1 (Invitrogen Corp.) and pEGFP.C2 (CLONTECH Laboratories). All plasmids generated by PCR were sequenced at the UNC sequencing facility.

Cell Culture and Transfections

Type II MDCK cells, human bronchial epithelial 16HBE14o− (Haws et al., 1992), and human colonic epithelial T84 cells were maintained in DME-F12 (Life Technologies, Gaithersburg, MD) + 10% fetal clone serum (Hyclone) at 37°C and 95% humidity. Primary cultures of human nasal epithelium were prepared as described previously (Matsui et al., 1998). To generate stable 16HBE14o− cell lines expressing GFP.YAP65 or GFP.YAP65/−4, cells were transfected as described (Chen et al., 1998) and grown in media containing 400 μg/ml G418. Drug-resistant colonies were expanded, and clonal lines were selected by fluorescence microscopy and Western blot analysis using rabbit anti-GFP antisera (Chemicon International, Inc.).

Analysis of YAP65, c-Yes, and c-Src Expression

Confluent monolayers of cultured cells were washed with PBS (50 mM $NaH_2PO_4 \cdot H_2O$, 150 mM NaCl, pH 7.4) and lysed in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% deoxycholic acid, and 5 mM EDTA (RIPA) containing 250 μM sodium orthovanadate and a cocktail of protease inhibitors. Lysates were cleared by centrifugation at 40,000 *g* for 20 min at 4°C. Protein concentrations were determined using the BCA assay kit (Pierce Chemical Co.), and 30 μg of cell lysate was electrophoresed on 10% SDS-PAGE gels and transferred to Immobilon-P (Millipore). Western blot analysis was performed using rabbit anti-YAP65 (1:1,000), mouse anti-Yes (1:1,000), mouse anti-Src (1:1,000), or rabbit EBP50 antisera as described (Short et al., 1998).

Immunohistochemical Analyses

16HBE14o− cell monolayers were fixed in 4% paraformaldehyde for 5–20 min at room temperature and blocked in 4% nonfat dry milk, 2 mg/ml BSA, 0.1% Triton X-100 for 3 h (for 16HBE14o−), or 10–20% normal goat serum (primary cultures). Cultures were incubated in primary antisera for 4–12 h in PBS containing 2 mg/ml BSA. After washing in PBS + 0.1% Triton X-100, the filters were incubated in Alexa 488– (1:200; Mo-

lecular Probes) or Texas red–conjugated (1:500; Jackson ImmunoResearch Laboratories, Inc.) secondary antiserum diluted in PBS + 2 mg/ml BSA. The samples were analyzed using a Leica TCS-NT confocal microscope as described (Chen et al., 1998).

In Vitro Binding Assays

Biotinylated peptides or GST fusion proteins (10 μg) were immobilized on streptavidin agarose or glutathione agarose as described (Short et al., 1998). To assess binding between immobilized peptides or GST fusion proteins and in vitro translated proteins, plasmids containing the appropriate insert were used as templates for coupled in vitro transcription/translation (Promega TNT), in the presence of 2 μCi [^{35}S]methionine (NEN). 5 μl of radiolabeled protein was incubated with the affinity resins in binding buffer + 0.1% Triton X-100 for 3 h at 4°C. The samples were centrifuged to collect the beads, and the beads were washed three times in TEE containing 1 M NaCl + 1% Triton X-100 followed by three washes in binding buffer + 1% Triton X-100. The bound fractions were eluted from the beads and the supernatants were precipitated with ice-cold acetone. Bound and unbound fractions were analyzed by SDS-PAGE and phosphorimage analysis. For competition experiments, GST-EBP50 was immobilized on glutathione agarose beads, and the beads were incubated for 2 h in the presence of competing peptide, before the addition of in vitro translated protein or cell lysate.

In some experiments, affinity resins were incubated with whole cell lysates prepared from 16HBE14o− cells. The cell lysates were prepared in RIPA buffer containing orthovanadate and protease inhibitors as described above, and ~200 μg of cell lysate diluted in binding buffer + 0.1% Triton X-100 was incubated with the affinity resin for 3 h at 4°C. The beads were washed as described above, proteins were eluted from the beads, and fractionated by SDS-PAGE. Bound fractions were analyzed by Western blotting using the appropriate antisera. All binding assays were performed at least three times with similar results.

Coimmunoprecipitation Experiments

Cells were rinsed in PBS and lysates were prepared in RIPA buffer. Rabbit anti-EBP50, preimmune sera, or purified normal rabbit IgG (2 μg) was prebound to protein A beads in binding buffer + 0.1% Triton X-100. Beads were washed in the same buffer + 1% Triton X-100. Cell lysates were added and the samples were incubated for 4–12 h at 4°C in RIPA buffer (500 μl final volume). The samples were centrifuged to collect the beads, and washed three times with RIPA buffer. Bound proteins were analyzed by blotting with the appropriate antisera or subjected to in vitro kinase assays. Kinase activity was measured after addition of a Src family substrate peptide derived from p34^{cdc2} (KVEKIGEGTYGVVYK; Upstate Biotechnology) according to the manufacturer's instructions.

Results

EBP50 and YAP65 Associate In Vitro

While NHE3 is known to associate with EBP50 via a nontraditional PDZ interaction (Yun et al., 1997; Weinman et al., 1998), there are no known proteins that associate with PDZ2 via a COOH-terminal interaction. We identified YAP65 (Fig. 1) as a candidate protein to associate with PDZ2 of EBP50. In addition to the potential PDZ interaction motif at the extreme COOH terminus, YAP65 contains a proline-rich sequence that mediates association with SH3 domains and a WW domain (Sudol, 1994; Sudol et al., 1995). Computer-based structural analyses of the human YAP65 protein sequence using the Coils algorithm (Lupas et al., 1991) with a window size of 28, predicted a single coiled coil from residues 259–292 with the probability of 1.

We first asked whether EBP50 and YAP65 were able to associate in vitro. We immobilized GST or GST-EBP50 on glutathione agarose beads, incubated the affinity resins with whole cell lysates prepared from 16HBE14o− cells,

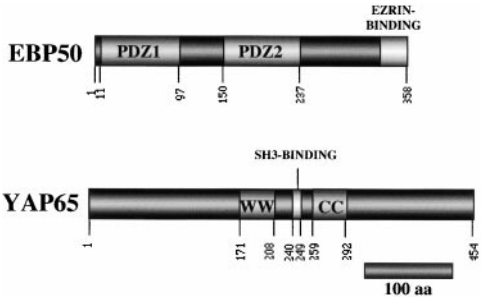

Figure 1. Domain organization of EBP50 and YAP65. EBP50 contains two PDZ domains and a COOH-terminal ezrin-binding domain. Human YAP65 contains a WW domain (residues 171–208), an SH3-binding motif (residues 240–249), a predicted coiled coil (CC; residues 259–292), and a COOH-terminal PDZ interaction motif.

and examined the bound and unbound fractions by Western blot using YAP65 antisera (Sudol, 1994). Endogenous YAP65 bound to the GST-EBP50 beads, but not to GST beads, indicating that EBP50 and YAP65 are capable of interacting (Fig. 2 A).

To determine whether the COOH terminus of YAP65 was involved in the interaction, we immobilized biotinylated peptides corresponding to the COOH-terminal 10 amino acids of YAP65 (Fig. 2 B, YAPwt) on streptavidin beads, and incubated the immobilized peptide with whole

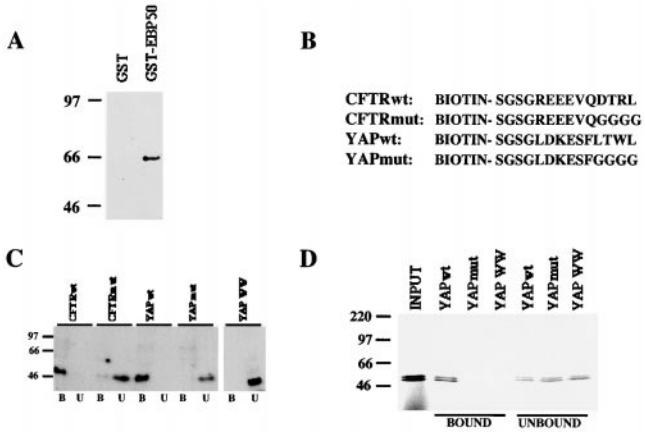

Figure 2. In vitro association of EBP50 and YAP65. (A) Lysates of 16HBE14o− cells (100 μg) were incubated with immobilized GST or GST-EBP50 (10 μg), washed, and analyzed by SDS-PAGE followed by immunoblotting with rabbit anti-YAP65 antisera. (B) Primary sequence of biotinylated peptides used in binding and competition experiments. (C) Biotinylated peptides (10 μg) were immobilized on streptavidin agarose and incubated with 16HBE14o− cell lysates (100 μg). Bound (B) and unbound (U) fractions were electrophoresed on 10% SDS-PAGE gels and analyzed by immunoblotting using rabbit anti-EBP50 antisera. (D) Radiolabeled EBP50 was generated by coupled in vitro transcription/translation in the presence of [^{35}S]methionine and incubated with immobilized wild type (CFTRwt, YAP65wt), mutant COOH-terminal (CFTRmut, YAP65mut) or YAP65 WW domain peptides (10 μg). Bound and unbound fractions were analyzed by SDS-PAGE and phosphorimage analysis.

cell lysates prepared from 16HBE14o− cells. We performed parallel incubations using CFTRwt peptide affinity resins (Fig. 2 B) since we previously showed that CFTR and EBP50 associate in vitro (Short et al., 1998). We found that EBP50 bound to both CFTR and YAP65 COOH-terminal peptides and was depleted from the cell lysate (Fig. 2 C). Affinity resins containing the YAP65 WW domain or mutant YAP65 peptide (Fig. 2 B; YAPmut) bound no EBP50. Therefore, the interaction between YAP65 and EBP50 is specific, and requires the COOH terminus of YAP65.

To determine whether the association between YAP65 and EBP50 is direct, we tested the ability of YAP65 peptides to bind radiolabeled, in vitro translated EBP50. YAPwt peptide, but not YAPmut or WW domain peptides, bound labeled EBP50 (Fig. 2 D). The YAPwt peptide also bound GST-EBP50, but not GST, in blot overlay assays (not shown), further supporting our conclusion that EBP50 and YAP65 directly associate.

Distribution and Localization of YAP65

EBP50 is expressed in many cells, and especially high levels are found in epithelial tissues (Reczek et al., 1997). We analyzed the expression of YAP65 protein in MDCK cells, human intestinal T84, and two cultured human airway epithelial cell lines, 16HBE14o− and CalU3 cells. YAP65 protein is expressed in whole cell lysates prepared from each of the cell lines tested (Fig. 3 A). Furthermore, similar to ezrin and EBP50, a significant amount of YAP65 protein was found in the particulate fraction of these cells (Fig. 3 B).

We showed previously that ezrin and EBP50 are preferentially accumulated at the apical surface of human airway epithelia (Short et al., 1998); however, the subcellular localization of YAP65 has not been previously studied. Therefore, we compared the distributions of ezrin, EBP50, and YAP65 in 16HBE14o− cells, a clonal cell culture model system derived from human bronchus. In our culture system, 16HBE14o− cells form monolayers with transepithelial resistances of >400 ohm·cm^2 and exhibit vectorial ion transport properties when grown on permeable filter supports. These cells also exhibit an asymmetric distribution of proteins including ZO-1 (tight junctions) and ezrin (apical; Fig. 3 C). Thus, 16HBE14o− cell monolayers provide a well polarized airway epithelial cell culture system to examine the distribution of proteins in EBP50 complexes.

Cells that were grown on transwell filters for 7–10 d after confluence were stained with rabbit antisera directed against EBP50 or YAP65. Both proteins were preferentially accumulated at the apical membrane (Fig. 3 C). The amount of each protein present in the apical compartment increased when cells were maintained on transwell filters for increasing lengths of time (not shown), suggesting that the recruitment of EBP50 and YAP65 to the membrane is partially dependent upon cell polarization.

We also examined the distribution of YAP65 in well differentiated primary human nasal epithelial cells grown on transwell supports at the air–liquid interface for 40–50 d. These cultures recapitulate the morphology of the well differentiated pseudostratified epithelia lining the normal

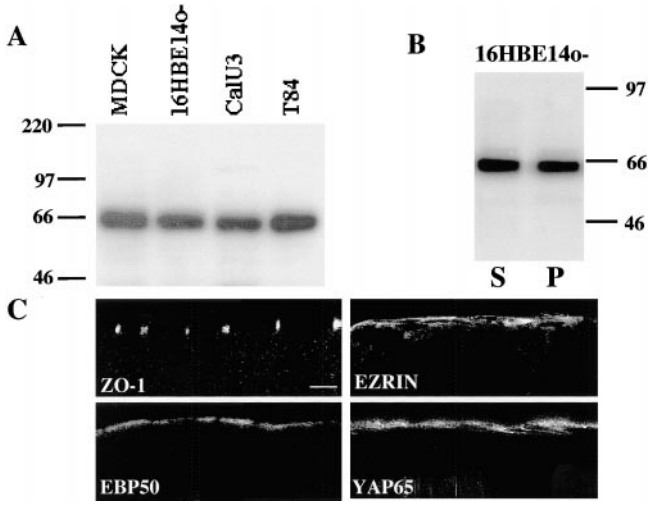

Figure 3. Expression and subcellular localization of YAP65 in cultured cells. (A) 30 μg of total cell lysate were analyzed by Western blot analysis using rabbit anti-YAP65 antisera. (B) 16HBE14o− cells were lysed in TEE and centrifuged to obtain soluble (S) and particulate (P) fractions. 20 μg of each fraction was electrophoresed on 10% SDS-PAGE gels and analyzed using YAP65 antisera. (C) 16HBE14o− cells, grown on transwell filters, were fixed in paraformaldehyde, blocked, and incubated with mouse anti-ezrin (1:300), rat anti-ZO-1 (1:200), rabbit anti-EBP50 (1:500), or rabbit anti-YAP65 (1:300). After washes, the cultures were incubated with appropriate Texas red– or FITC-conjugated secondary antisera and XZ sections were analyzed by confocal microscopy. Bar, 10 μm.

airway surface, and ciliated columnar epithelial cells and mucin-producing goblet cells are present (Fig. 4 A; Matsui et al., 1998). As observed in 16HBE14o− cells, ezrin and EBP50 were preferentially accumulated at the apical membrane and their distributions overlapped (Fig. 4 B). The overlap between ezrin and EBP50 was observed in ciliated and nonciliated cells, and did not significantly vary from culture to culture. YAP65 was also accumulated at the lumenal surface of cultured primary nasal epithelial cells, in a pattern that largely overlapped the distribution of apical actin (Fig. 4 C). In all cultures examined the distributions of YAP65 and ezrin partially overlapped (Fig. 4 D), but regions where the distributions of the two proteins were juxtaposed, but not overlapping, were also observed. In addition, we also observed that in some cultures, YAP65 was more broadly distributed in the subapical space (see Fig. 9 A). Nonetheless, these localization studies clearly establish that ezrin, EBP50, and YAP65 are co-distributed in the apical compartment of human nasal and bronchial epithelial cells.

Determination of the YAP65 Binding Site in EBP50

In vitro data indicate that the COOH terminus of CFTR binds with high affinity to the first PDZ domain of EBP50 (Hall et al., 1998b; Short et al., 1998; Wang et al., 1998). To determine whether YAP65 bound to the same or different sites on EBP50, we immobilized GST-EBP50 on affinity resins and incubated the resins with radiolabeled YAP65 or the COOH terminus of CFTR (CFTR-CT; residues

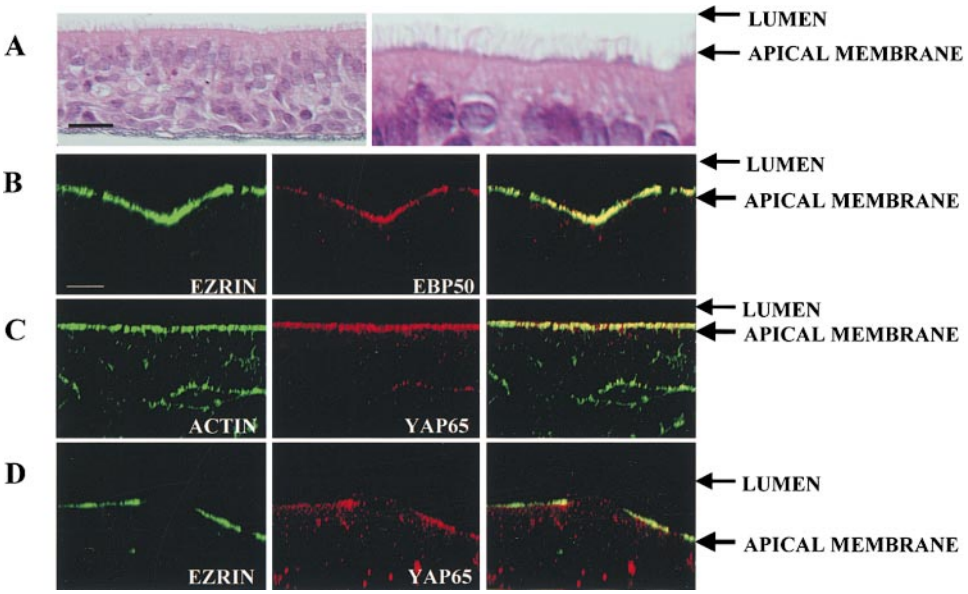

Figure 4. Localization of YAP65, ezrin, and EBP50 in primary well differentiated human nasal epithelia. (A) Well differentiated, pseudostratified primary cultures of human nasal epithelia were counterstained with hemotoxylin and eosin and analyzed by confocal microscopy. A higher magnification of the lumenal surface is shown on the right. (B) Primary human nasal epithelial cultures were incubated with antiserum directed against ezrin (1:300) and EBP50 (1:200). (C) Primary human nasal epithelial cultures were incubated with antiserum directed against YAP65 (1:200) and labeled with 70 nM FITC-conjugated phalloidin to visualize actin. (D) Primary human nasal epithelial cultures were incubated with antiserum directed against ezrin (1:300) and YAP65 (1:200). After washes, the cultures (B–D) were incubated with appropriate Texas red– or FITC-conjugated secondary antisera and mounted for confocal analysis. Bar, 10 μm.

1,150–1,480 of human CFTR). As expected, both proteins bound to the GST-EBP50 affinity resins (Fig. 5 A, lanes 1 and 6). The interaction between CFTR-CT and EBP50 was blocked by the addition of 400-nM CFTRwt peptide, but not by the CFTRmut peptide lacking the terminal DTRL (Fig. 5 A, lanes 2 and 3). Likewise, we found that the YAPwt peptide competed for binding between GST-EBP50 and radiolabeled YAP65, whereas the YAPmut peptide lacking LTWL failed to compete (Fig. 5 A, lanes 9 and 10). Binding between GST-EBP50 and CFTR-CT was not blocked by 400-nM YAP65 peptide (Fig. 5 A, lane 4), nor was binding between GST-EBP50 and radiolabeled YAP65 blocked by addition of CFTR COOH-terminal peptide (Fig. 5 A, lane 7). Thus, CFTR and YAP65 do not compete for binding to EBP50 in vitro, suggesting that the two proteins associate with EBP50 via distinct sites. However, both YAP65 and CFTR (Short et al., 1998) appear to bind EBP50 with high affinity. When increasing amounts of YAP65 COOH-terminal peptide ranging from 1–500 nM were added to YAP65-EBP50 binding assays, association was inhibited ~50% by 100-nM competing peptide and completely abolished by 500-nM peptide (Fig. 5 B).

Our competition experiments indicate that YAP65 and CFTR associate with EBP50 via distinct binding sites. To directly determine which PDZ domain of EBP50 was involved in binding YAP65, we tested the association between in vitro translated full-length YAP65 and equal amounts of GST-EBP50 fusion proteins. We found that YAP65 associated with GST-EBP50 and GST-PDZ2 of EBP50, but not with GST or GST-PDZ1 (Fig. 5 C). Taken together, these data indicate that YAP65 preferentially associates with the second PDZ domain of EBP50.

YAP65 and EBP50 Associate In Vivo

Our in vitro binding assays demonstrate that YAP65 and EBP50 are capable of interaction, and our localization studies place the two proteins within the apical compartment of airway epithelial cells. Therefore, we sought to determine whether EBP50 and YAP65 associate in cells. We incubated whole cell lysates from 16HBE14o− cells with normal rabbit IgG or with rabbit antisera directed against EBP50, collected immune complexes on protein A agarose, and analyzed the immunoprecipitates using ezrin or YAP65 antisera. As previously reported (Reczek et al., 1997), ezrin was found in EBP50 immunoprecipitates but not in control immunoprecipitates (Fig. 6 A). Moreover, YAP65 was not associated with normal rabbit IgG, but a significant fraction of the YAP65 expressed in the 16HBE14o− cells was associated with EBP50 (Fig. 6 A). By comparing the amount of YAP65 present in the EBP50 immunoprecipitates to the amount present in the whole cell lysate, we estimate that ~20% of the total YAP65 expressed in these cells was associated with EBP50.

To determine whether the COOH terminus of YAP65 mediated its association with EBP50 in cells, we generated stable 16HBE14o− cell lines expressing GFP fused in-frame with YAP65 (GFP-YAP65) or YAP65 lacking the last four amino acids shown to be involved in association with EBP50 (GFP-YAP65/–4). Cell lines expressing similar levels of GFP-YAP65 and GFP-YAP65/–4 were selected; immunoblot analyses using YAP65 antisera indicated that the expression of chimeric protein relative to endogenous YAP65 is ~1:1 (not shown). Neither GFP chimera was found to associate with the beads when lysates were incubated with protein A agarose in the absence of antibody, or when samples were immunoprecipitated with normal rabbit IgG (Fig. 6 B). In agreement with the co-immunoprecipitation experiments from nontransfected cells, a significant fraction of GFP-YAP65 was present in EBP50 immunoprecipitates (Fig. 6 B). In contrast, GFP-YAP65/–4 did not immunoprecipitate together with

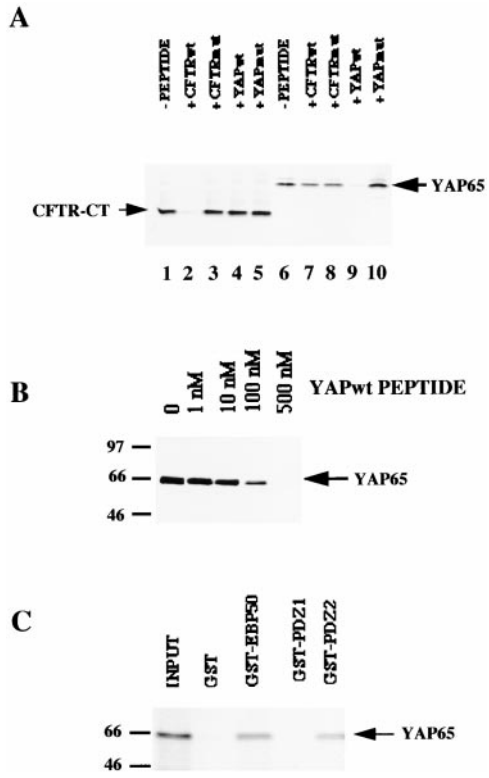

Figure 5. Identification of YAP65 binding site in EBP50. (A) GST-EBP50 (10 μg) was immobilized on beads and incubated with (+ peptide) or without (− peptide) 400 nM COOH-terminal peptides as indicated. Radiolabeled CFTR-CT or full-length YAP65 were added to binding reactions. Bound proteins were extensively washed in binding buffer, eluted, and analyzed by SDS-PAGE and applied to phosphorimage screens. (B) Immobilized GST-EBP50 (10 μg) was incubated with radiolabeled full-length YAP65 in the presence of increasing concentrations of YAP65wt peptide. Bound proteins were analyzed as described in A. (C) 10 μg of GST, GST-EBP50, GST-PDZ1, or GST-PDZ2 were immobilized on glutathione agarose beads and incubated with radiolabeled YAP65. After washing in buffers containing 1 M NaCl, bound proteins were eluted from the beads and analyzed as described in A.

EBP50. Taken together, our data indicate that EBP50 and YAP65 associate in a stable complex in cells, and that the association requires an intact YAP65 COOH terminus.

To determine whether the association with EBP50 is involved in compartmentalizing YAP65 at the apical membrane, we compared the distributions of GFP-YAP65 and GFP-YAP65/−4 in the stable cell lines. Like endogenous YAP65, GFP-YAP65 was concentrated in the apical compartment of 16HBE14o− cells (Fig. 6 C). In contrast, the GFP-YAP65/−4 mutant protein was not accumulated at the apical cell surface, indicating that the EBP50–YAP65 interaction is required for the proper localization of YAP65 in airway epithelia. Surprisingly, the GFP-YAP65/−4 mutant protein was preferentially associated with the lateral cell surfaces of the transfected cells. These data indicate YAP65 can associate with proteins or lipids on the lateral cell surface, but that the full-length protein localizes to the apical compartment because of its high affinity interaction with EBP50.

Association of EBP50 and YAP65 with Src Family Kinases

YAP65 was originally cloned based on its ability to associate in vitro with the SH3 domain of c-Yes, a nonreceptor tyrosine kinase (NRTK) of the Src family (Sudol, 1994). These data raise the intriguing possibility that, by binding YAP65, EBP50 functions to recruit c-Yes or other NRTKs to the apical cell surface where they may play key regulatory roles. To begin to explore the functional significance of the EBP50–YAP65 interaction, we determined whether EBP50 protein complexes also contained NRTK activity when isolated in vitro. We immobilized GST-EBP50 fusion proteins on glutathione agarose, and incubated the affinity resins with lysates prepared from 16HBE14o− cells. After incubation, the affinity resins were extensively washed to remove unbound proteins and incubated with γ-[^{32}P]ATP in the presence of the Src family kinase–specific substrate peptide, p34^{cdc2}. Under these conditions, we observed significant levels of Src family kinase activity associated with GST-EBP50 and GST-PDZ2, whereas background levels of activity were associated with GST and GST-PDZ1 (Fig. 7).

Since we previously showed that YAP65 associated with GST-EBP50, and that this interaction was mediated by the COOH terminus of YAP65, we next determined whether the association between EBP50 and Src family kinases was mediated by YAP65. We preincubated GST-EBP50 affinity resins with 400-nM YAPwt peptide, and then measured the levels of Src family kinase activity associated with the GST-EBP50 affinity resins. We found that incubation of GST-EBP50 affinity resins with the YAPwt peptide completely blocked the association between GST-EBP50 and the Src family kinase activity (Fig. 7, GST-EBP50 + YAPwt). These data are consistent with the hypothesis that YAP65 serves as an adaptor protein to recruit Src family kinases to EBP50 complexes.

Our immunoblot analyses of whole cell lysates prepared from 16HBE14o− and CalU3 cells indicate that both c-Src and c-Yes are expressed in these cells (Fig. 8 A). Although previous in vitro binding assays suggest that YAP65 preferentially associates with the c-Yes SH3 domain (Sudol 1994), binding between YAP65 and Src family kinases has not been carefully examined in cells. To determine whether the NRTK activity associated with GST-EBP50 (Fig. 7) is due to association between EBP50, YAP65, and c-Yes, we incubated 16HBE14o− cell lysates with immobilized GST-EBP50 fusion proteins. c-Yes immunoreactivity was easily visualized in the bound fraction of GST-EBP50 and GST-PDZ2 affinity resins (Fig. 8 B). In contrast, no c-Yes was found to associate with GST or with GST-PDZ1 (Fig. 8 B). In addition, we performed duplicate GST-PDZ pull-down experiments using antisera to both c-Yes and c-Src. As expected, a significant fraction of c-Yes was associated with GST-PDZ2 but not GST (Fig. 8 C). In contrast, while c-Src was easily detected in the input sample, we observed minimal binding to GST and GST-PDZ2 (Fig. 8 C). Taken together these data indicate that YAP65 specifically mediates the association between EBP50 and c-Yes, but not c-Src. Furthermore, when EBP50 immunoprecipitates were blotted with antisera to c-Yes, we found a significant fraction of the c-Yes ex-

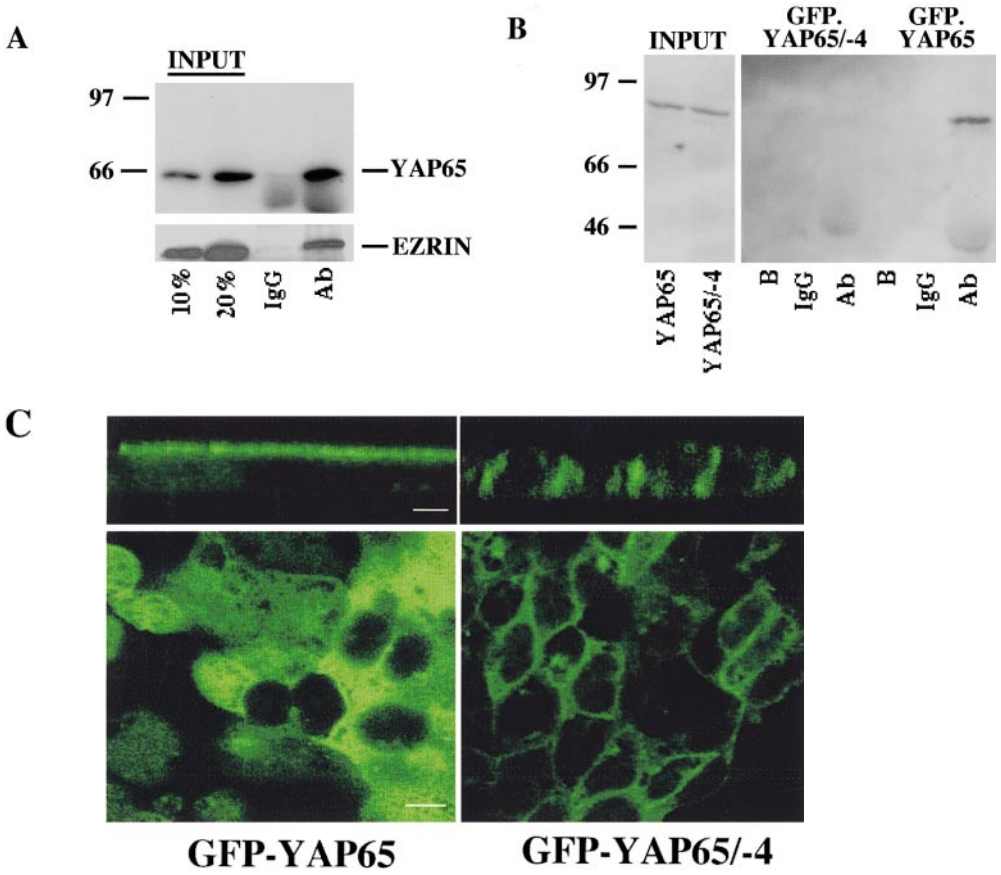

Figure 6. In vivo association of YAP65 and EBP50. (A) Whole cell lysates from wild-type 16HBE14o− cells were prepared in RIPA buffer and ~300 μg of total protein was immunoprecipitated using EBP50-specific antisera (Ab) or normal rabbit IgG. Bound proteins were extensively washed in RIPA buffer, eluted, electrophoresed on 10% SDS-PAGE and blotted with rabbit anti-YAP65 (1:1,000) or mouse anti-ezrin (1:1,000) antisera. Input: 10% or 20% of the material added into each binding reaction. (B) Lysates from 16HBE14o− cells stably expressing GFP-YAP65 or GFP-YAP65/−4 (200 μg) were immunoprecipitated using EBP50-specific antisera as described in A. Bound proteins were electrophoresed on 10% SDS-PAGE and blotted with mouse anti-GFP (1:1,000). Input: 20% of the material added into each binding reaction. B, protein A beads alone; IgG, normal rabbit IgG; and Ab, rabbit anti-EBP50. (C) 16HBE14o− cells stably expressing GFP-YAP65 or GFP-YAP65/−4 were grown to confluence on transwell filters, fixed in 4% paraformaldehyde, and analyzed by confocal microscopy. At least two independent clones were analyzed for each cell line. Bars, 10 μm.

pressed in 16HBE14o− cells in the immunoprecipitates. (Fig. 8 D). By comparing the amount of c-Yes in the whole cell lysate to the amount found in the EBP50 immunoprecipitates, we conclude that ~15–20% of the c-Yes is contained within EBP50 protein complexes in 16HBE14o− cells. Taken together, the data indicate that EBP50, YAP65, and c-Yes stably associate in cells.

Distribution of Yes Kinase in Airway Epithelia

Previous immunohistochemical studies suggest that c-Yes is localized at the apical membrane of intestinal and tracheal epithelium (Zhao et al., 1990). c-Yes is also enriched at adherens junctions in liver and in some cultured cells (Tsukita et al., 1991), and in lipid-insoluble complexes at the apical cell surface of MDCK cells (Sargiacomo et al., 1993). Our biochemical assays indicating a stable association between EBP50, YAP65, and c-Yes are consistent with these immunohistochemical studies; however, we also found that a significant fraction of the c-Yes expressed in 16HBE14o− cells was not associated with YAP65. Therefore, we used confocal microscopy to compare the distributions of EBP50, YAP65, and c-Yes in our cell culture model systems. In all cultures examined, c-Yes was visualized at the apical cell surface, although some of the protein was present in other cellular compartments and on intracellular vesicles (Fig. 9). The degree of colocalization varied from culture to culture, suggesting that the state of differentiation or other unknown factors influence the distribution of c-Yes. The partial overlap between EBP50, YAP65, and c-Yes is consistent with our biochemical results, indicating that a significant fraction of these proteins are stably associated in cells.

YAP65 May Function to Target c-Yes to the Apical Cell Surface

Although our work confirms that YAP65 and c-Yes stably associate in cells, the function of this interaction is not yet known. Many protein kinases and phosphatases are restricted to distinct subcellular compartments by association with specific targeting proteins (Pawson and Scott, 1997; Schillace and Scott, 1999). Therefore, we considered the possibility that one function of the interaction between YAP65 and c-Yes is to target c-Yes to the apical cell surface. We used confocal microscopy to compare the distribution of c-Yes in 16HBE14o− cells stably expressing GFP.YAP65 and GFP.YAP65/−4. Consistent with the localization in wild-type 16HBE14o− cells (Fig. 9), c-Yes was accumulated at the apical membrane in cells stably overexpressing GFP.YAP65 (Fig. 10 A). In contrast, in cells stably overexpressing GFP.YAP65/−4, a significant

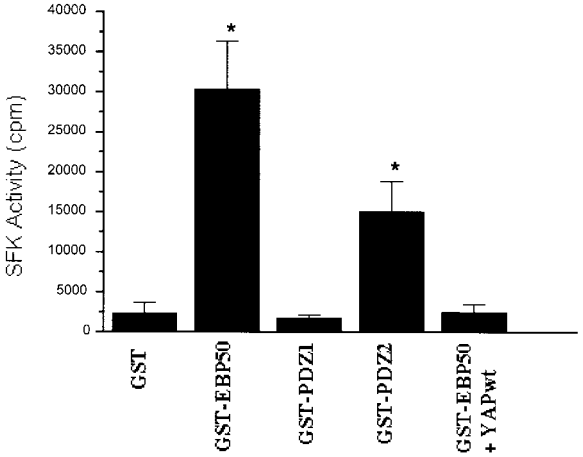

Figure 7. Association of EBP50 with Src family kinase activity in 16HBE14o− cells. GST fusion proteins (10 μg), immobilized on glutathione agarose, were incubated with ~200 μg of 16HBE14o− cell lysate. For competition experiments, immobilized GST-EBP50 was preincubated with 400 nM YAPwt peptide for 3 h before addition of lysates. Beads were washed, and kinase activity was measured by adding an exogenous Src family substrate, $p34^{cdc2}$ and 1 μCi γ-[^{32}P]ATP. Statistical analysis was performed using the *t* test (*$P < 0.01$; $n = 3$).

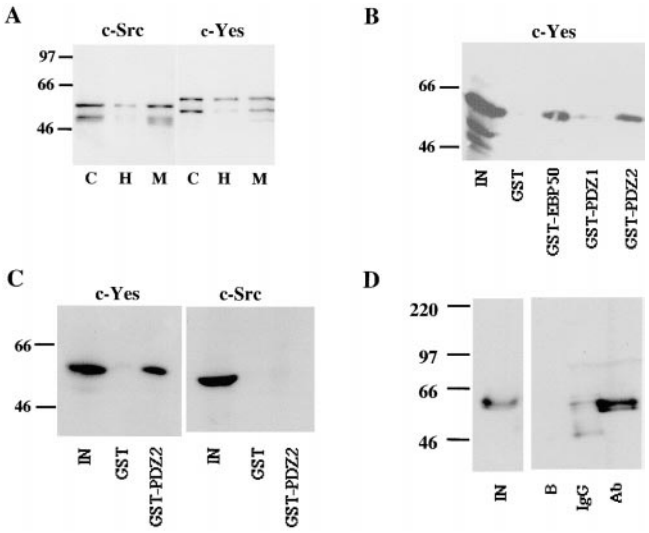

Figure 8. Association of YAP65 and c-Yes in 16HBE14o− cells. (A) 30 μg of total cell lysate from CalU3 (C), 16HBE14o− (H), or MDCK (M) cells was electrophoresed on 10% SDS-PAGE gels and analyzed by immunoblotting with c-Src (1:1,000−) or c-Yes (1:1,000) antisera as noted above the blot. (B and C) Immobilized GST fusion proteins (10 μg) were incubated with 16HBE14o− cell lysates (~200 μg) diluted in binding buffer. After extensive washing in binding buffer, bound proteins were eluted from the beads, electrophoresed on 10% SDS-PAGE and analyzed by immunoblotting using c-Yes (1:1,000) or c-Src (1:1,000) antisera. Input (In): 20% (B) or 25% (C) of the material added into each binding reaction. (D) Lysates from wild-type 16HBE14o− cells (~200 μg) were immunoprecipitated using EBP50-specific antisera (Ab), rabbit IgG, or protein A beads alone (B). Bound proteins were washed and electrophoresed on 10% SDS-PAGE and blotted with mouse anti–c-Yes antisera. Input (In): 20% of the material added into each reaction.

amount of c-Yes was redistributed to the lateral cell borders along with the mutant GFP-YAP65/–4 protein (Fig. 10 B). Some c-Yes was still detected at the apical membrane of cells overexpressing GFP.YAP65/–4, and was likely due to association with endogenous YAP65 or other apical membrane components. These data build on our conclusion that YAP65 and c-Yes stably associate in cells and further suggest that this interaction functions to target c-Yes to the apical cell surface.

Discussion

EBP50 and the related protein E3KARP (NHE3 kinase A regulatory protein), are known to regulate the activity of the Na^+/H^+ exchanger NHE3 (Weinman et al., 1995; Yun et al., 1997; Lamprecht et al., 1998). This regulation involves direct binding between an internal sequence within the cytoplasmic COOH terminus of NHE3 and residues 149–358 of EBP50 (Weinman et al., 1998; Yun et al., 1998). EBP50 binds ezrin, and ezrin is known to bind the regulatory subunit of PKA in overlay assays (Dransfield et al., 1997). Therefore, one hypothesis is that EBP50 recruits PKA to the apical membrane in close proximity with NHE3 (Lamprecht et al., 1998; Yun et al., 1998). While this model is attractive, there are no conclusive data establishing a role for ezrin as an A kinase anchoring protein in cells.

In addition to NHE3, CFTR and the βAR also directly associate with EBP50 (Hall et al. 1998a,b; Short et al., 1998; Wang et al., 1998). While the function of the CFTR–EBP50 interaction is not yet known, binding of β_2AR to EBP50 is implicated in regulation of cellular pH by modulation of NHE3. Specifically, in fibroblasts, agonist activation of βARs stimulated association of the receptor with EBP50, and prevented EBP50 from regulating NHE3 (Hall et al., 1998b). However, these experiments have not been replicated in polarized cells, where NHE3 is known to reside at the apical cell surface (Biemesderfer et al., 1997; Janecki et al., 1998). Furthermore, NHE3 is not expressed in airway epithelial cells, indicating that the functions of EBP50 may be tissue-specific. EBP50 may function to target proteins to the apical cell surface, and may also serve as a scaffold to organize apical membrane proteins into regulatory complexes. A logical approach to elucidating the function of EBP50 is the identification of additional proteins in EBP50 protein complexes.

Our data establish that YAP65 associates with EBP50 via the COOH terminus, and the COOH terminus of YAP65 is necessary and sufficient for association with EBP50 (Figs. 2, 5, and 6). In addition, pull-down assays using the YAP65wt peptide and GST fusion proteins of PDZ1 and PDZ2, clearly demonstrated preferential binding of YAP65 to PDZ2 (Fig. 5 C). Furthermore, the YAP65 COOH-terminal peptide was unable to compete for binding between GST-EBP50 and CFTR-CT (Fig. 5 A). These results strongly support the conclusion that YAP65 is recruited to EBP50 complexes by association with PDZ2 of EBP50.

Our localization studies clearly demonstrate that endogenous YAP65 is preferentially localized at the apical mem-

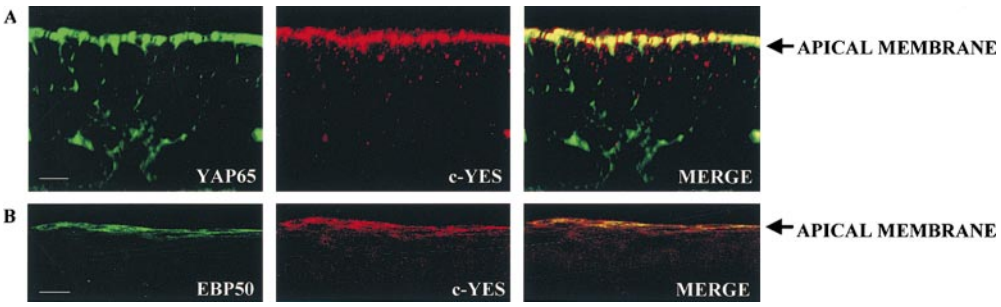

Figure 9. Localization of c-Yes in airway epithelial cells. (A) Well differentiated primary human nasal epithelia and (B)16HBE14o− cells grown to confluence on transwell filters were fixed and blocked as described in Materials and Methods followed by incubation in antisera specific for c-Yes (1:100), YAP65 (1:200), or EBP50 (1:200). After washes, the cultures were incubated with appropriate Texas red– or FITC-conjugated secondary antisera and analyzed on the confocal microscope. Bars, 10 μm.

brane of 16HBE14o− cells and in well differentiated primary cultures from human nasal epithelial cells (Figs. 3 and 4). The expression of GFP-YAP65 chimeras in 16HBE14o− cells supports our localization studies, since GFP-YAP65 was also accumulated at the apical membrane (Fig. 6 C). Furthermore, our results indicate that YAP65 is tethered at the apical membrane by association with EBP50, since GFP-YAP65/−4, which lacks the PDZ2 interaction motif, was no longer localized at the apical membrane (Fig. 6 C). Since we were able to coimmunoprecipitate EBP50 and GFP-YAP65, but not GFP-YAP65/−4 (Fig. 6 B), we conclude that association with EBP50 functions to recruit YAP65 to the apical compartment in polarized cells. Together with the overexpression studies, our ability to coimmunoprecipitate endogenous EBP50 and YAP65 from 16HBE14o− cells (Fig. 6 A) suggests that the two proteins stably associate. However, we cannot rule out the possibility that the binding of YAP65 to other apical membrane PDZ proteins is also involved in the targeting of YAP65. Two additional PDZ proteins, E3KARP and PDZK1, share significant sequence identity with the EBP50 PDZ domains (Yun et al., 1995; Kocher et al., 1998), and both proteins may associate with YAP65 in vitro (Kultgen, P., and S.L. Milgram, unpublished results). Thus, it is possible that YAP65 associates with several apical membrane PDZ proteins including EBP50, and that together these proteins are responsible for the compartmentalization of YAP65 in the subapical compartment. The generation of additional reagents to carefully examine the expression and subcellular distributions of E3KARP and PDZK1 will be needed to resolve this question.

Additional Proteins in EBP50–YAP65 Complexes

YAP65 contains multiple protein–protein interaction domains, and the binding of YAP65 and EBP50 would allow for the recruitment of additional proteins to the subapical compartment. If YAP65 functions as a dimer, as suggested by the presence of the predicted coiled coil, YAP65 could facilitate the formation of EBP50 multimers, thus, generating a larger protein complex. Dimerization of YAP65 might also be involved in mediating, or regulating, its association with other proteins. Since YAP65 contains one or two WW domains (depending on alternative mRNA splicing; Espanel and Sudol, 1999) it is likely that additional proteins associate with YAP65. Two novel proteins, WBP1 and WBP2, have been identified which associate with the first WW domain of human YAP65 in vitro (Chen et al., 1997; Linn et al., 1997). Thus, it will be critical to determine whether one or both of these proteins associate with YAP65 in cells, and what functional roles these interactions may play.

YAP65 was cloned using an anti–idiotype antibody directed against the protooncogene c-Yes, a member of the Src family of NRTKs. Although the two proteins were clearly shown to associate in vitro, our GST-EBP50 pulldown experiments demonstrate for the first time that YAP65 and c-Yes stably associate in cells (Fig. 8 B). There is considerable overlap in the distributions of YAP65 and c-Yes in our cell culture model systems (Fig. 9). Furthermore, a significant fraction of the endogenous c-Yes expressed in 16HBE14o− cells was redistributed from the apical membrane in cells overexpressing GFP-YAP65/−4 (Fig. 10). Collectively, these data suggest that in epithelial

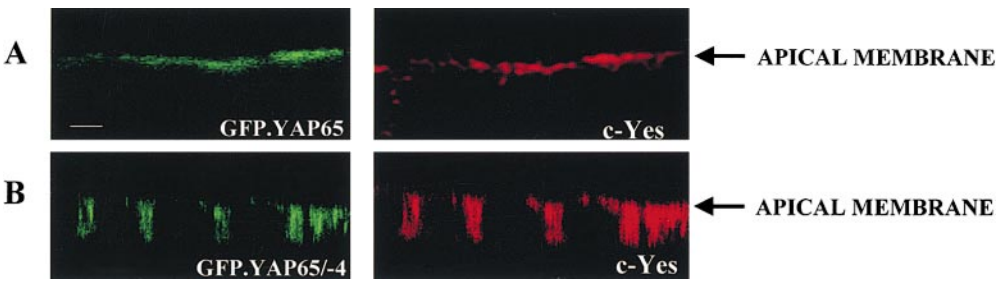

Figure 10. Stable association of YAP65 and c-Yes in airway epithelial cells. 16HBE14o− cells stably expressing (A) GFP-YAP65 or (B) GFP-YAP65/−4 were grown to confluence on transwell filters, fixed in 4% paraformaldehyde, stained with mouse anti–c-Yes antisera (1:100) and analyzed by XZ scanning confocal microscopy. Bar, 10 μm.

cells one function of YAP65 is to target c-Yes to the apical cell surface. Thus, similar to AKAPs (A kinase anchoring proteins) and RACKs (receptors for activated C kinase), which interact with protein kinase A and C, respectively (Mochly-Rosen et al., 1991; Colledge and Scott, 1999; YAP65 may restrict the activity and enhance the specificity of c-Yes in epithelia. In addition, it is possible that YAP65 may also modulate the activity of the Src family kinase by a yet unknown mechanism. Additional experiments, both in cells and in vitro, will be required to fully understand the function of these interactions.

By comparing the amount of c-Yes in the input sample, and the amount found associated with GST-EBP50 affinity columns and coimmunoprecipitations, we estimate that 15–20% of the c-Yes expressed in 16HBE14o− cells was copurified together with YAP65. These results are not surprising since many other proteins are known to associate with Src family kinases, and these protein associations are regulated dynamically by extracellular stimuli (for review see Thomas and Brugge, 1997). Although we clearly see colocalization of EBP50, YAP65, and c-Yes in the well differentiated primary nasal epithelial cultures and 16HBE14o− cells, we also find c-Yes in other cellular compartments (Fig. 9). In kidney epithelial cells stably overexpressing epitope-tagged ezrin, c-Yes was found in ezrin immunoprecipitates (Crepaldi et al., 1997). Since ezrin and EBP50 are known to associate (Reczek et al., 1997), we have not ruled out the possibility that a portion of the activity associated with EBP50 was actually bound by ezrin. However, a direct association between ezrin and c-Yes has not been demonstrated.

In biochemical assays, YAP65 was also shown to bind the SH3 domain of c-Src and c-Yes, although the affinity for c-Yes was greater (Sudol, 1994). Since c-Src is expressed in 16HBE14o− cells (Fig. 8 A), we also probed GST-EBP50 affinity resins with c-Src antisera, but did not find evidence for c-Src associated with EBP50. Therefore, our data suggest that the YAP65–EBP50 complex specifically associates with c-Yes at the apical cell surface. However, YAP65 likely associates with multiple proteins that contain SH3 domains, so it would not be surprising to find small amounts of c-Src contained within the EBP50 protein complexes. Our GFP-YAP65 cell lines will be very useful for determining whether additional proteins associate with the YAP65 proline-rich motif.

Potential Functions of EBP50-YAP65-Yes Kinase Interactions

Our previous work and the new results reported here identify several members of the EBP50 protein complex, including an adaptor protein (YAP65) and an NRTK (c-Yes). Although we focused on airway epithelia, EBP50, YAP65 and c-Yes are widely expressed in epithelia, and the makeup of EBP50 protein complexes will differ based on the specific genes expressed in each epithelium. One possible function for apical membrane c-Yes is modulation of ion channels. Src family kinases play a critical role in modulating ion transport through voltage and ligand-gated ion channels (for review see Thomas and Brugge, 1997). Furthermore, Src family kinase–mediated regulation of ion transport may be facilitated by interactions of the kinases with submembranous scaffolding proteins. For example, the association of PSD95 and Fyn in neurons facilitates tyrosine phosphorylation of the *N*-methyl-D-aspartate receptor (Tezuka et al., 1999). In addition, the direct binding of p56lck to hDlg is thought to be involved in modulation of Shaker Type Kv1.3 K^+ channels (Hanada et al., 1997). Src family kinases may be involved in the regulation of Na^+/H^+ exchanger function (Krump et al., 1997; Tsuganezawa et al., 1998). Since EBP50 directly binds NHE3 and indirectly associates with c-Yes, it is intriguing to speculate that these interactions are important for modulation of NHE3. Although NHE3 is not expressed in airway epithelial cells (Brant et al., 1995), Src family kinases may modulate the activity of other apical membrane conductances in these cells. Since exogenously applied c-Src can regulate the gating of CFTR in heterologous expression systems (Fischer and Machen, 1996), it will be important to determine whether c-Yes functions as a regulator of CFTR in vivo.

Src family kinases are also involved in the control of gene expression (Boyer et al., 1997; Li et al., 1998). In addition, a recent report indicates that YAP65 may serve as a transcriptional coactivator in some cells, and exogenously expressed YAP65 was observed in the nucleus of NIH 3T3 fibroblasts (Yagi et al., 1999). Although we do not see YAP65 in the nucleus of polarized airway epithelial cells grown on permeable supports, it is possible that YAP65 is capable of translocation from the cytoplasm to the nucleus in response to extracellular stimuli. Such regulation may be analogous to that of β-catenin, a protein at the epithelial adherens junction, that translocates from the cell membrane to the nucleus to regulate gene expression (Huber et al., 1996; Simcha et al., 1998). Therefore, proteins contained within EBP50 complexes may be involved in transducing signals from the apical cell surface to the nucleus in response to external stimuli. Further characterization of proteins contained within EBP50 protein complexes in different cell types and the identification of extracellular stimuli that modulate the composition of these complexes will be critical for understanding the regulation of membrane proteins present at the apical cell surface in epithelia.

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